



Presence of nitrates and nitrites in fresh cow milk from milk machines during winter and summer period in the city Zagreb and Zagreb County area

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ABSTRACT

Introduction: The aim of the study was to determine the nitrites and nitrates content in fresh cow's milk samples from milking machines, and to determine whether their amount is affected by the season of sampling (summer/winter).

Methods: The methodology used was analytical transversal method at 2-time points. All milk samples were sampled at milk machines from the City of Zagreb and Zagreb County, during the summer and winter months in 2020. A total of 40 milk samples were sampled, with 20 samples in each monitored period (summer/winter). A high-performance liquid chromatography with a diode array detector was used to identify and quantify concentrations of the nitrate and the nitrite content. The results were processed using descriptive statistics and the statistics of differences.

Results: The analysis determined the range of nitrate content from 1.28 mg/kg to 19.71 mg/kg and the range of nitrite content was from 0.49 mg/kg to 3.42 mg/kg in milk samples. The mean result of nitrates in fresh cow's milk samples in the summer period was 9.12 mg/kg and in the winter period of 3.88 mg/kg. The mean value of nitrite in the summer was 1.23 mg/kg, while the mean value of nitrite in the winter period was 1.48 mg/kg.

Conclusion: The research has shown that the nitrate and the nitrite levels in local fresh cow's milk sampled in milk machines do not exceed the maximum allowable intake. The obtained results confirmed that the amounts of nitrates and nitrites in the milk samples differ significantly during the winter and summer periods and differences are depending on the location of the milk machine.

Keywords: Fresh cow's milk; nitrates; nitrites; HPLC; public health

INTRODUCTION

Milk is a natural liquid of pale yellowish color and sweet taste, produced in the mammary glands of female mammals, and represents an indispensable food in the 1st days of newborns because it contains the most important nutrients needed for their growth and functional development (1). Milk contains all nutrients in favorable proportions, such as fats, proteins and carbohydrates, as well as protective substances such as vitamins and minerals, while in the highest proportion, it contains water (about 87%), which makes it the most complete natural liquid known (2). Milk must not contain residues of veterinary medicinal products or other residues, contaminants, or substances that may

impair human health. It must have its characteristic odor, taste, and color, without the addition of water. As well, it must not contain more than 400,000 somatic cells in 1 ml of milk, since their higher number indicates possible bad health condition of females known as the mastitis and should be milked for a period of <30 days before calving the cow and not earlier than 10 days after calving (3).

The form of nitrate is a natural form of nitrogen and is an integral part of the nitrogen cycle in the environment and agriculture, due to the increasing use of nitrogen-based fertilizers, which penetrate through the soil to groundwater. Nitrates enter the human body where they can adversely affect our health in various ways (4). Plants also take nitrates from the soil and then convert them to produce their own proteins by photosynthesis, and if nitrate intake is higher than protein conversion, nitrates may accumulate. Increased nitrate intake can occur because of the lack of plant energy due to low levels of sunlight, wilting, or

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retardation of plant development and due to low ambient temperatures. Furthermore, after a long drought, the plant begins to grow again with the first rains, so the nitrate intake is faster. Some types of soil, even without the addition of nitrogen fertilizers, contain a high proportion of nitrogen and easily bring nitrates to the plant due to their acidity (5).

Nitrates, which the cow enters the body through food or water, are converted into ammonia and are converted into a bacterial protein in the rumen. Since nitrates are converted to nitrites faster than nitrites to ammonia, the nitrites are accumulated in the rumen when nitrates are consumed higher than normal. By absorbing nitrites into the bloodstream, the hemoglobin is converted to the methemoglobin and the oxygen transport is disabled, which can lead to the death of the animal. The concentration of nitrates in plants is variable, so the occurrence of nitrate poisoning is not easy to predict. Fodder plants, that is, leaves and stems, accumulate more nitrates than cereals and other concentrates. Since the diet of ruminants includes a higher percentage of fodder plants and the intake of nitrates in the animal's body is higher (6).

In food products, such as meat, fish and milk and dairy products, sodium and potassium salts of nitrates, and nitrites are used as additives, to improve the taste, color, and appearance and extend the shelf life of the product. The use of these additives (E 249-252) keeps the color of the meat red and improves the taste. The nitrite salts, due to their antimicrobial properties, are also used to prevent the growth of pathogen microorganisms, such as *Clostridium botulinum*, a bacterium that causes botulism. In vegetables, nitrates are found naturally which can enter the food chain as an environmental pollutant in water, due to intensive and excessive use in agriculture. According to European Food Safety Agency (EFSA) research, consumers' exposure to nitrates from food additives is estimated not to pose a serious health risk, as it is <5% of total nitrate exposure in food, thus not exceeding safe intake levels. However, the safe intake level can be exceeded if the natural presence of nitrates in food as well as environmental pollution is considered. As children consume larger amounts of food in relation to their body weight, we can assume that there is a risk of exceeding the acceptable daily intake. Although nitrites have been linked to the production of nitrosamine compounds, which are known to be potential carcinogens, EFSA claims that there are no major health concerns if used at approved levels. However, nitrite levels not intentionally present in foods, such as from contaminated environment, can contribute to the production of nitrosamines and such levels could lead to potential health problems (7).

The International Agency for Research on Cancer has linked the presence of nitrates and nitrites in foods to an increased risk of gastrointestinal cancer and methemoglobinemia in newborns and young children. Chamandust et al. concluded that the presence of nitrates and nitrites in food, such as milk, can be considered as dangerous compounds during oxidative and reducing state in the gastrointestinal tract, since the nitrites formed during reduction of bacteria in saliva and stomach acid in reaction with secondary amines produce carcinogenic N-nitrosamine compounds. Consequently, the nitrites are much more toxic compounds than nitrates and can be classified as potential

carcinogens (8), although there is an evidence to suggest that nitrates may have a therapeutic effect and are used in the treatment of a number of cardiovascular diseases (9).

Changes in the distribution and sale of milk in the EU have led to increased use of milk machines, devices in which consumers can buy fresh cow's milk, usually using their own packaging. The reasons for the popularity of such sales explain the results of a survey conducted by the Croatian Food Agency (HAH), where 38% of respondents believe that milk from milking machines is healthier, 29% of respondents believe that buying milk from milking machines directly helps the milk producer, while 25% of respondents believe that milk from milking machines is better and of better taste, identifying it with milk consumed in childhood (10).

The effects of any dangerous substance on health depend on the dose, duration, mode of exposure, personal qualities, and lifestyle (11).

The primary (main) goal of this study was to conduct sampling of fresh cow's milk samples at milk machines available to consumers to determine whether and in what quantities the sampled milk contains nitrites and nitrates, and whether the established maximum allowable levels exceed.

The additional goal of the research was to determine whether the obtained values of nitrite and nitrate differ according to the seasons (winter–summer) and to determine whether there are differences in the amounts of nitrite and nitrate in milk depending on the location of the milk machine.

METHODS

A total of 40 fresh milk samples in sterile 1 l plastic bottles were collected at the same milking machines: 20 samples during the winter period and 20 samples during the summer period. Random sampling was performed at the milk machines in several locations of the City of Zagreb and the Zagreb County area. Immediately after sampling, samples are delivered into the laboratory in portable refrigerator at +4°C. On arrival, the samples were marked with a unique analytical number and were stored in a freezer at -18°C until the start of nitrite/nitrate analysis. All samples were analyzed within 6 weeks of sampling.

Before the start of the verification of the analytical procedure, a procedure to control the purity of the laboratory glass containers and chemicals was performed that will be used during the verification of the analysis, to obtain accurate and reliable results. All dishes were washed with 0.1 M HCl solution and followed by deionized water. In parallel with the test samples, a "blank test" was performed in which the presence of nitrites and nitrates was not determined.

After homogenization of the sample, 1 g ± 0.05 g of the sample was weighed into a 50 mL volumetric flask and ~20 mL of redistilled water was added. The sample was extracted on a magnetic stirrer for 5 min at ~80°C, then the flask was refilled up to 50 mL. All samples were centrifuged at 4500 rpm for 10 min and were filtered through 0.22 µm GMF Whatman chromatographic glass filter and microfiber in a sampler bottle.

All analyzes were performed using a high-performance liquid chromatography technique (HPLC Agilent 1200) with

a diode array detector to quantify the content of nitrites and nitrates salts. The HPLC used an C18 column (length of 250 mm), an internal diameter of 4.6 mm, filled with Strong Anion Exchange filler with a particle size of 5 μm . The column temperature was set at 40°C. Samples were recorded at a wavelength of ($\lambda = 214 \text{ nm}$) and a column temperature of 30°C with a flow of 1 ml/min of potassium dihydrogen phosphate (10 g/L) that was used for the mobile phase, with the pH = 3 value.

The method validation parameters are described in Table 1. The chromatogram of nitrite and nitrate standards has been presented in Figure 1.

The results obtained were processed using IBM SPSS statistics v.25. Since the sample volume was <50, the Shapiro–Wilk test was applied. Since it was found that the results do not have a normal distribution in the further analysis, a non-parametric Mann–Whitney U-test was applied, and the obtained results were considered statistically significant if their value was of $p < 0.05$.

RESULTS

The first group of results refers to seasonal differences in the concentration of nitrate and nitrite in fresh drinking cow's milk samples, sampled at the milk machines of the City of Zagreb and Zagreb County, that are shown in Tables 2 and 3.

Table 2 shows the results of the distribution of nitrites and nitrates in samples during summer and winter. The nitrate levels range from 1.28 mg/kg to 19.71 mg/kg, while the nitrite concentration ranges from 0.49 mg/kg to 3.42 mg/kg.

Table 3 shows that the mean values of nitrates which in the summer were 28.80 mg/kg, while in the winter were 12.20 mg/kg. From these results, it can be concluded that there is a statistically significant difference ($p < 0.05$) related to the seasonal differences in nitrate concentration in fresh milk at all locations of milking machines.

The second group of results in Tables 4–6 shows that the results related to seasonal differences in the levels of nitrates and nitrites at certain research sites.

Table 4 shows that the level of nitrates in fresh drinking cow's milk sampled at the locations of the City of Zagreb milking machines ranged from 1.89 mg/kg to 13.60 mg/kg, and nitrites from 0.49 mg/kg to 1.85 mg/kg, while in the milk sampled in the Zagreb County the range of nitrates level was from 1.28 mg/kg to 19.71 mg/kg, and nitrites ranged from 0.90 mg/kg to 3.42 mg/kg. Taking into account evident seasonal differences and the sampling area, it was statistically determined that for the obtained nitrate results, there is a statistically significant difference ($p < 0.05$).

In the third group of results, Tables 7–9 show the seasonal differences, where the groups are divided according to location.

Table 7 shows that the results of nitrates and nitrites according to the seasonal period divided according to the locations of the milking machines. The level of nitrates in fresh drinking cow's milk during the summer in the City of Zagreb ranged between 3.69 mg/kg and 13.60 mg/kg, and

TABLE 1. Validation parameters of the method applied

Parameter	Acceptance criterion obtained
Results scattering	3.71%
Recovery	99.2±5%
Repeatability of measurement	0.11%
Repeatability of sample preparation	2.71%
Linearity	K≥0.999
Intermediate precision	3.33%
Limit of quantification	1 mg/kg

TABLE 2. The nitrite and nitrate distribution in fresh cow's milk during summer and winter

Parameter	Number of samples	Mean value	Stand deviation	Minimum	Maximum
Nitrites (NO_2^-)	40	1.3563	0.60398	0.49	3.42
Nitrates (NO_3^-)	40	6.4970	4.17475	1.28	19.71

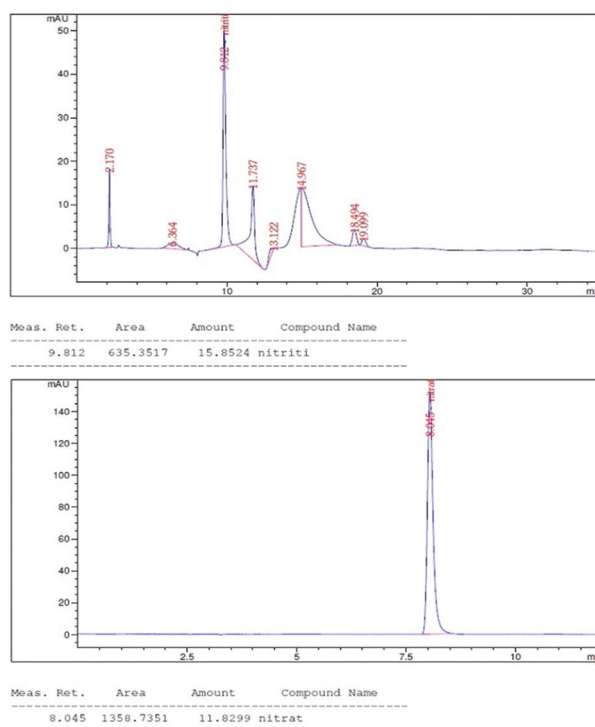


FIGURE 1: The chromatogram of nitrite and nitrate standards

in the Zagreb County from 4.52 mg/kg to 19.71 mg/kg. The nitrite content in milk ranged between 0.78 mg/kg and 1.52 mg/kg in the City of Zagreb and from 1.10 mg/kg to 1.80 mg/kg in the Zagreb County. According to the obtained results, it can be concluded that there is no statistically significant differences in the nitrate and the nitrite content during the summer period, related to location ($p > 0.05$).

Table 8 shows the seasonal differences of nitrite and nitrate content in fresh cow's milk samples during the summer period related to location, while Table 9 shows the seasonal differences of nitrite and nitrate in fresh cow's milk samples during the winter period related to location. It is evident that in the winter period around the City of Zagreb, the level of nitrates ranged from 1.89 mg/kg to 7.20 mg/kg, while the level of nitrites ranged from 0.49 mg/kg and 1.85 mg/kg. In the Zagreb County, the values for nitrates

TABLE 3. Seasonal differences in nitrite and nitrate concentrations in fresh cow's milk samples (sampled at all milk machine locations)

Parameter	Season	Number of samples	Mean value	Mean values sum	U	Z	Sig	<i>p</i>
Nitrites (NO ₂ ⁻)	Summer	20	19.68	393.5	183.5	-0.446	0.655	>0.05
	Winter	20	21.33	426.5				
Nitrates (NO ₃ ⁻)	Summer	20	28.80	576.0	34.0	-4.49	0.000	<0.05
	Winter	20	12.20	244.0				

TABLE 4. Overview of the distribution of nitrites and nitrates in fresh cow's milk during summer and winter at the locations of milking machines of the City of Zagreb and Zagreb County

Parameter	Location	Number of samples	Mean value	Standard deviation	Minimum	Maximum
Nitrates (NO ₃ ⁻)	City of Zagreb	20	6.0810	3.11924	1.89	13.60
Nitrites (NO ₂ ⁻)	City of Zagreb	20	1.0495	0.33996	0.49	1.85
Nitrates (NO ₃ ⁻)	Zagreb County area	20	6.9130	5.06758	1.28	19.71
Nitrites (NO ₂ ⁻)	Zagreb County area	20	1.6630	0.65963	0.90	3.42

TABLE 5. Seasonal differences in nitrite and nitrate content in fresh cow's milk at the locations of the City of Zagreb's milk machines

Parameter	Season	Number of samples	Mean value	Mean value sum	U	Z	Sig.	<i>p</i>
Nitrate (NO ₃ ⁻)	Summer	10	14.30	143.00	12.0	-2.873	0.004	<0.05
	Winter	10	6.70	67.00				
Nitrite (NO ₂ ⁻)	Summer	10	12.05	120.50	34.5	-1.172	0.241	>0.05
	Winter	10	8.95	89.50				

TABLE 6. Seasonal differences in nitrite and nitrate content in fresh cow's milk at the locations of Zagreb County milk machines

Parameter	Season	Number of samples	Mean value	Mean value sum	U	Z	Sig	<i>p</i>
Nitrates (NO ₃ ⁻)	Summer	10	15.10	151.00	4.00	-3.477	0.001	<0.05
	Winter	10	5.90	59.00				
Nitrites (NO ₂ ⁻)	Summer	10	8.10	81.00	26.0	-1.815	0.07	>0.05
	Winter	10	12.90	129.00				

TABLE 7. Overview of results according to the seasonal period of nitrite and nitrate in fresh cow's milk, related to milk machine locations

Season	Parameter	Location	Number of samples	Mean value	Standard deviation	Minimum	Maximum
Summer	Nitrates (NO ₃ ⁻)	City of Zagreb	10	7.9340	3.13089	3.69	13.60
		Zagreb County area	10	10.3010	5.11276	4.52	19.71
	Nitrites (NO ₂ ⁻)	City of Zagreb	10	1.1150	0.25614	0.78	1.52
		Zagreb County area	10	1.3480	0.24899	1.10	1.80
Winter	Nitrates (NO ₃ ⁻)	City of Zagreb	10	4.2280	1.76288	1.89	7.20
		Zagreb County area	10	3.5250	1.60187	1.28	5.87
	Nitrites (NO ₂ ⁻)	City of Zagreb	10	0.9840	0.41091	0.49	1.85
		Zagreb County area	10	1.9780	0.79754	0.90	3.42

TABLE 8. Seasonal differences of the nitrite and the nitrate content in fresh cow's milk samples during the summer period, related to locations

Parameter	Location	Number of samples	Mean value	Mean value sum	U	Z	Sig	<i>p</i>
Nitrates (NO ₃ ⁻)	City of Zagreb	10	9.60	96.00	41.00	-0.68	0.529	>0.05
	Zagreb County area	10	11.40	114.00				
Nitrites (NO ₂ ⁻)	City of Zagreb	10	8.60	86.00	31.0	-1.438	0.159	>0.05
	Zagreb County area	10	12.40	124.00				

TABLE 9. Seasonal differences of the nitrite and the nitrate content in fresh cow's milk samples during the winter period, related to locations

Parameter	Location	Number of samples	Mean value	Mean value sum	U	Z	Sig	<i>p</i>
Nitrates (NO ₃ ⁻)	City of Zagreb	10	11.70	117.00	38.00	-0.907	0.393	>0.05
	Zagreb County area	10	9.30	93.00				
Nitrites (NO ₂ ⁻)	City of Zagreb	10	6.60	66.00	11.0	-2.948	0.002	<0.05
	Zagreb County area	10	14.40	144.00				

ranged from 1.28 mg/kg to 5.87 mg/kg, while the values for nitrites ranged from 0.90 mg/kg to 3.42 mg/kg. Considering the level of nitrites in fresh cow's milk samples

according to locations, it is to conclude that there is a statistically significant difference during the winter period ($p < 0.05$).

DISCUSSION

The maximum permitted nitrate and nitrite content in fresh drinking cow's milk are not set by "Commission Regulation (EC) No 1881/2006 of December 19, 2006 setting maximum levels for certain contaminants in foodstuffs" and may pose a potential problem related to their concentrations if the national authorities does not conduct systematic control and monitoring of the target type of food (12). However, although there are no maximum levels of nitrate and nitrite in fresh drinking cow's milk are set, the European Food Safety Authority (EFSA) in its research recommends acceptable daily nitrate intake for the humans, which for adults are from 0 to 3.7 mg/kg of body weight per day (13,14). It is important to consider that nitrate intake is based on total nitrate exposure from all sources during the day, which includes water, air, and cigarette smoke in addition to food (14,15).

Yeh et al. conducted a study in Taiwan on the nitrate and the nitrite content in milk and milk powder samples. The study was conducted on 100 milk samples by ion chromatography technique. Milk samples were taken from four categories: Fortified milk powder, plain milk powder, fresh milk, and milk in aseptic packaging. Since powdered milk is intended for the adult population, enriched with nutraceutical drugs, the result was an increased concentration of nitrate compared to powdered milk for infants and young children. The concentration of nitrates in whey milk samples was higher on average than the concentration of nitrate in infant milk by 200 ppm. Due to the higher amount in water in fresh milk samples, the lowest level of nitrate content was detected. The highest level of nitrate content was detected in whey hydrolyzate samples, which was 48.23 ppm. According to Yeh, dairy products and foods intended for children can contain nitrate concentrations up to 1760 ppm. In this study, the highest nitrates concentration was 417.7 ppm, which is below the detection limit. Yeh concluded that even the highest proportion of nitrate proven in the tested samples does not exceed the daily acceptable intake (16).

The obtained data should be placed in the context of the research conducted by Brkić et al. (2012) regarding the concentrations of nitrates in green leafy vegetables (lettuce, kale, spinach, chard, cabbage, and arugula). Vegetable samples were collected in the spring and autumn in four biggest cities in Croatia: Zagreb, Rijeka, Split and Osijek. A total of 200 samples of green leafy vegetables were sampled, 100 samples in spring and 100 samples in autumn. To determine nitrates, they used the technique of high-performance liquid chromatography (HPLC) with UV detection. The lowest nitrate levels found were 603.0 mg/kg in kale sampled in the spring, while the highest level of 972.2 mg/kg was found in chard, over the same period. In the autumn period, the lowest level of nitrate proven in chard was 1024.7 mg/kg, and the highest level of 2013.1 mg/kg was found in spinach.

With the entry into force of the new EC Regulation, in the autumn period, sampling of arugula plant (rocket) was carried out, in which the concentration of nitrate was 4354.9 mg/kg. Brkić et al. concluded that there are statistically significant differences at all sampling sites, considering a seasonal difference (17).

It is important to notice that the composition of milk is influenced by several factors, but primarily depends on the type of animal and its condition (lactation stage, udder disease, etc.) (10). Since nitrates does not mainly accumulate in the animal's body, but are converted to ammonia in the rumen, one of the most important factors in preventing nitrate poisoning is total daily nitrate intake, with total nitrate intake being a critical problem. Undersander et al. claim how nitrate poisoning can be prevented by dangerous levels of nitrate in the food can be safe for consumption if consuming only half the meal normative. They also explain, how the same amount of nitrate in one dose (one meal) can be toxic, while the same level of nitrate divided into several smaller meals can be completely safe. Therefore, if the same amount of toxic level is given gradually in meals over a long period, it is likely that toxic effects will not occur, while they would certainly occur if the toxic level is given occasionally, in one meal. In conclusion, they note that it is important to determine the concentration of nitrate in suspicious feeds, but also water, which can also be a crucial factor in poisoning, if contains high levels of nitrates (6).

According to the EFSA, further research is needed to create a complete picture of the carcinogenic effects of nitrates and nitrites on the human body. Equally, it is important to point out that new research on the conversion of nitrates to nitrites in human saliva, which results in the production of methemoglobin, would be of great benefit in the future, all for the protection of human health, especially children (7).

The importance of research is also seen in the fact that raw milk vending machines exist not only in Italy and Germany but also in Austria, Croatia, Czech Republic, Denmark, France, Greece, Ireland, Lithuania, the Netherlands, and other countries including African countries like Kenya (18,19).

Given the results obtained and the proven differences in the incidence of nitrates and nitrites in milk over two season periods, the future research should investigate in depth the possible impact of diet on observed seasonal differences.

CONCLUSION

Based on the obtained results of the presence of nitrates and nitrites in milk from milking machines, it can be concluded that the research objectives have been met.

The research has confirmed that the levels of nitrate and nitrite in fresh cow's milk from the dairy do not exceed the maximum allowable intake in accordance with the recommended daily intake of nitrate in the human body.

Furthermore, the research has also confirmed that the statistically significant differences in nitrate level were found at all milk machine locations, and a statistically significant difference in nitrite incidence was found between nitrate and nitrite levels in the analyzed samples.

Although the concentrations of nitrates and nitrites in fresh cow's milk do not exceed the recommended daily intake, it should be taken into account that the intake of nitrates and nitrites in the human body is significant also through dairy products, meat and meat products, fruits and vegetables, and drinking water, as well as through exposure to tobacco

smoke, and that is why the intake should be limited from all sources.

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